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| (21) International Application Number: PCT/US97/12919 (22) International Filing Date: 28 July 1997 (28.07.97) (30) Priority Data: 08/699,040 19 August 1996 (19.08.96) US (71) Applicant: UNIVERSITY OF MASSACHUSETTS [US/US]; A Public Institution of Higher Education of the Common- wealth of Massachusetts, represented by its Amherst Cam- pus, Office of Vice Chancellor for Research at Amherst, Amherst, MA 01002 (US). (72) Inventors: ROBL, James; 196 Old Enfield, Belchertown, MA 01002 (US). CIBELLI, Jose; 166 Village Park, Amherst, MA 01002 (US). STICE, Steven, L.; 468 Amherst Road, Belchertown, MA 01007 (US). (74) Agent: TESKIN, Robin, L.; Burns, Doane, Swecker & Mathis, L.L.P., P.O. Box 1404, Alexandria, VA 22313-1404 (US). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> | |
| (54) Title: EMBRYONIC OR STEM-LIKE CELL LINES PRODUCED BY CROSS SPECIES NUCLEAR TRANSPLANTATION | | | |
| (57) Abstract | | | |
| <p>An improved method of nuclear transfer involving the transplantation of donor cell nuclei into enucleated oocytes of a species different from the donor cell is provided. The resultant nuclear transfer units are useful for the production of isogenic embryonic stem cells, in particular human isogenic embryonic or stem cells. These embryonic or stem-like cells are useful for producing desired differentiated cells and for introduction, removal or modification, of desired genes, e.g., at specific sites of the genome of such cells by homologous recombination. These cells, which may contain a heterologous gene, are especially useful in cell transplantation therapies and for <i>in vitro</i> study of cell differentiation.</p> | | | |

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days post-surgically as a precautionary measure (Cefilofur 1Hcl 50 mg/cc @ 1cc/100 pounds). Immediately following surgery a single injection intramuscularly or under the kidney capsule of Flunixin Meglumine @ 1cc/100 pounds may be given to control pain and swelling at the surgical site. If teratoma formation does not occur at the paralumbar fascia, other sites may be analyzed, i.e., subcutaneously.

It is expected that "same animal" stem cells will survive in the recipient (donor of nucleus) animal in contrast to "different animal" stem cells, or survive at least better or longer depending on the cytotoxic T cell response or other immune reaction to foreign mitochondrial peptides. Furthermore, it is expected that cells from all three germ layers, i.e., ectoderm, mesoderm, and endoderm, will be observed in "same animal" teratomas.

EXAMPLE 2

This example was designed to test teratoma formation in an immune-compromised animal model. This example is relevant to the methods whereby cloned, nuclear transfer-generated cells from a patient in need of a transplant may be grown in a SCID mouse or other immune-compromised animal in order to generate differentiated cells for isolation and design of engineered tissues for transplant.

ES cells transfected with GFP were derived from two adult Holstein steers (two different ES cell lines were derived from each animal). ICMs were derived from 12-day-old blastocysts.

Cell preparation and injection procedure:

Cells were cut into pieces (sections of no more than about 100 cells each) and loaded into a 1 ml syringe, no more than 200 microliters each, and preferably 100 microliters.

ICMS were mechanically isolated and loaded into a 1-ml syringe 100 to 150 microliters.

Cells were kept at room temperature in HECM-Hepes.

Twenty-two-gauge needles were used for injection procedures. Cells were injected into the skeletal muscle of the hind leg of SCID mice.

| Mou | Treatment | Amount | Observatio |
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chimeras when introduced into preimplantation mouse embryos, thus demonstrating their pluripotency (Bradley et al., *Nature*, 309:255-256 (1984)).

In view of their ability to transfer their genome to the next generation, ES cells have potential utility for germline manipulation of livestock animals by using ES cells with or without a desired genetic modification. Moreover, in the case of livestock animals, e.g., ungulates, nuclei from like preimplantation livestock embryos support the development of enucleated oocytes to term (Smith et al., *Biol. Reprod.*, 40:1027-1035 (1989); and Keefer et al., *Biol. Reprod.*, 50:935-939 (1994)). This is in contrast to nuclei from mouse embryos which beyond the eight-cell stage after transfer reportedly do not support the development of enucleated oocytes (Cheong et al., *Biol. Reprod.*, 48:958 (1993)). Therefore, ES cells from livestock animals are highly desirable because they may provide a potential source of totipotent donor nuclei, genetically manipulated or otherwise, for nuclear transfer procedures.

Some research groups have reported the isolation of purportedly pluripotent embryonic cell lines. For example, Notarianni et al., *J. Reprod. Fert. Suppl.*, 43:255-260 (1991), report the establishment of purportedly stable, pluripotent cell lines from pig and sheep blastocysts which exhibit some morphological and growth characteristics similar to that of cells in primary cultures of inner cell masses isolated immunosurgically from sheep blastocysts. (Id.) Also, Notarianni et al., *J. Reprod. Fert. Suppl.*, 41:51-56 (1990) discloses maintenance and differentiation in culture of putative pluripotential embryonic cell lines from pig blastocysts. Further, Gerven et al., *Anim. Biotech.*, 6(1):1-14 (1995) disclose the isolation of embryonic cell lines from porcine blastocysts. These cells are stably maintained in mouse embryonic fibroblast feeder layers without the use of conditioned medium. These cells reportedly differentiate into several different cell types during culture (Gerven et al., *Id.*).

Further, Saito et al., *Roux's Arch. Dev. Biol.*, 201:134-141 (1992) report bovine embryonic stem cell-like cell lines cultured which survived passages for three, but were lost after the fourth passage. Still further, Handyside et al.,

however, that their cell lines resembled epithelial cells more than pluripotent ICM cells. (*Id.*)

Still further, Smith et al., WO 94/24274, published October 27, 1994, Evans et al, WO 90/03432, published April 5, 1990, and Wheeler et al, WO 94/26889, published November 24, 1994, report the isolation, selection and propagation of animal stem cells which purportedly may be used to obtain transgenic animals. Also, Evans et al., WO 90/03432, published on April 5, 1990, reported the derivation of purportedly pluripotent embryonic stem cells derived from porcine and bovine species which assertedly are useful for the production of transgenic animals. Further, Wheeler et al, WO 94/26884, published November 24, 1994, disclosed embryonic stem cells which are assertedly useful for the manufacture of chimeric and transgenic ungulates. Thus, based on the foregoing, it is evident that many groups have attempted to produce ES cell lines, e.g., because of their potential application in the production of cloned or transgenic embryos and in nuclear transplantation.

The use of ungulate ICM cells for nuclear transplantation has also been reported. For example, Collas et al., *Mol. Reprod. Dev.*, 38:264-267 (1994) disclose nuclear transplantation of bovine ICMs by microinjection of the lysed donor cells into enucleated mature oocytes. The reference disclosed culturing of embryos *in vitro* for seven days to produce fifteen blastocysts which, upon transferral into bovine recipients, resulted in four pregnancies and two births. Also, Keefer et al., *Biol. Reprod.*, 50:935-939 (1994), disclose the use of bovine ICM cells as donor nuclei in nuclear transfer procedures, to produce blastocysts which, upon transplantation into bovine recipients, resulted in several live offspring. Further, Sims et al., *Proc. Natl. Acad. Sci., USA*, 90:6143-6147 (1993), disclosed the production of calves by transfer of nuclei from short-term *in vitro* cultured bovine ICM cells into enucleated mature oocytes.

Also, the production of live lambs following nuclear transfer of cultured embryonic disc cells has been reported (Campbell et al., *Nature*, 380:64-68 (1996)). Still further, the use of bovine pluripotent embryonic cells in nuclear

transfer and the production of chimeric fetuses has also been reported (Stice et al., *Biol. Reprod.*, 54:100-110 (1996)); Collas et al, *Mol. Reprod. Dev.*, 38:264-267 (1994).

Also, there have been previous attempts to produce cross species NT units (Wolfe et al., *Theriogenology*, 33:350 (1990)). Specifically, bovine embryonic cells were fused with bison oocytes to produce some cross species NT units possibly having an inner cell mass. However, embryonic cells, not adult cells were used, as donor nuclei in the nuclear transfer procedure. The dogma has been that embryonic cells are more easily reprogrammed than adult cells. This dates back to earlier NT studies in the frog (review by DiBerardino, *Differentiation*, 17:17-30 (1980)). Also, this study involved very phylogenetically similar animals (cattle nuclei and bison oocytes). By contrast, previously when more diverse species were fused during NT (cattle nuclei into hamster oocytes), no inner cell mass structures were obtained. Further, it has never been previously reported that the inner cell mass cells from NT units could be used to form an ES cell-like colony that could be propagated.

Also, Collas et al (*Id.*), taught the use of granulosa cells (adult somatic cells) to produce bovine nuclear transfer embryos. However, unlike the present invention, these experiments did not involve cross-species nuclear transfer. Also, unlike the present invention ES-like cell colonies were not obtained.

Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of producing embryonic or stem-like cells. In particular, there exists a need for producing human embryonic or stem-like cells given their significant therapeutic and diagnostic potential.

In this regard, numerous human diseases have been identified which may be treated by cell transplantation. For example, Parkinson's disease is caused by degeneration of dopaminergic neurons in the substantia nigra. Standard treatment for Parkinson's involves administration of L-DOPA, which temporarily ameliorates the loss of dopamine, but causes severe side effects and ultimately

the nucleus of a human cell into an enucleated animal oocyte, preferably an ungulate enucleated oocyte.

It is another object of the invention to provide a novel method for producing human embryonic or stem-like cells which involves transplantation of nuclei of a human cell, e.g., a human adult cell into an enucleated human oocyte.

It is another object of the invention to provide embryonic or stem-like cells produced by transplantation of nuclei of an animal or human cell into an enucleated oocyte of a different species.

It is a more specific object of the invention to provide human embryonic or stem-like cells produced by transplantation of the nucleus of a human cell into an enucleated animal oocyte, preferably an ungulate enucleated oocyte.

It is another object of the invention to use such embryonic or stem-like cells for therapy or diagnosis.

It is a specific object of the invention to use such human embryonic or stem-like cells for treatment or diagnosis of any disease wherein cell, tissue or organ transplantation is therapeutically or diagnostically beneficial.

It is another specific object of the invention to use the embryonic or stem-like cells produced according to the invention for the production of differentiated cells, tissues or organs.

It is a more specific object of the invention to use the human embryonic or stem-like cells produced according to the invention for the production of differentiated human cells, tissues or organs.

It is another specific object of the invention to use the embryonic or stem-like cells produced according to the invention for the production of genetically engineered embryonic or stem-like cells, which cells may be used to produce genetically engineered or transgenic differentiated human cells, tissues or organs, e.g., having use in gene therapies.

It is another specific object of the invention to use the embryonic or stem-like cells produced according to the invention *in vitro*, e.g. for study of cell dif-

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel method for producing embryonic or stem-like cells, and more specifically human embryonic or stem-like cells by nuclear transfer or nuclear transplantation. In the subject application, nuclear transfer or nuclear transplantation or NT are used interchangeably.

As discussed *supra*, the isolation of embryonic or stem-like cells by nuclear transfer or nuclear transplantation has never been reported. Rather, previous reported isolation of ES-like cells has been from fertilized embryos. Also, successful nuclear transfer involving cells or DNA of genetically dissimilar species, or more specifically adult cells or DNA of one species and oocytes of another species has never been reported. Also, to Applicants' knowledge, there has never been reported a method for producing human embryonic or stem-like cells in tissue culture. Rather, the limited human fetal cells and tissues which are currently available must be obtained from spontaneous abortion tissues and from aborted fetuses.

Also, prior to the present invention, no one obtained embryonic or stem-like cells by cross-species nuclear transplantation.

Quite unexpectedly, the present inventors discovered that human embryonic or stem-like cells and cell colonies may be obtained by transplantation of the nucleus of a human cell, e.g., an adult differentiated human cell, into an enucleated animal oocyte, which is used to produce nuclear transfer (NT) units, the cells of which upon culturing give rise to human embryonic or stem-like cells and cell colonies. This result is highly surprising because it is the first demonstration of effective cross-species nuclear transplantation, i.e., the transplantation of cell nuclei from an animal or human cell, e.g., adult cell, into the enucleated egg of a different animal species, to produce nuclear transfer units containing cells which when cultured under appropriate conditions give rise to embryonic or stem-like cells and cell colonies.

Preferably, the NT units used to produce ES-like cells will be cultured to a size of at least 2 to 400 cells, preferably 4 to 128 cells, and most preferably to

a size of at least about 50 cells.

In the present invention, embryonic or stem-like cells refer to cells produced according to the present invention. The present invention refers to such cells as stem-like cells rather than stem cells because of the manner in which they are produced, i.e., by cross-species nuclear transfer. While these cells are expected to possess similar differentiation capacity as normal stem cells they may possess some insignificant differences because of the manner they are produced. For example, these stem-like cells may possess the mitochondria of the oocytes used for nuclear transfer.

The present discovery was made based on the observation that nuclear transplantation of the nucleus of an adult human cell, specifically a human epithelial cell obtained from the oral cavity of a human donor, when transferred into an enucleated bovine oocyte, resulted in the formation of nuclear transfer units, the cells of which upon culturing gave rise to human stem-like or embryonic cells and human embryonic or stem-like cell colonies. It is hypothesized by the present inventors that bovine oocytes and human oocytes must undergo maturation processes which are sufficiently similar to permit the bovine oocyte to function as an effective substitute or surrogate for a human oocyte.

Based on the fact that human cell nuclei can be effectively transplanted into bovine oocytes, it is reasonable to expect that human cells may be transplanted into oocytes of other species, e.g., other ungulates as well as other animals. In particular, other ungulate oocytes should be suitable, e.g. pigs, sheep, horses, goats, etc. Also, oocytes from other sources should be suitable, e.g. oocytes derived from other primates, amphibians, rodents, rabbits, etc. Further, using similar methods, it should be possible to transfer human cells or cell nuclei into human oocytes.

Therefore, in its broadest embodiment, the present invention involves the transplantation of an animal or human cell nucleus or animal or human cell into the enucleated oocyte of an animal species different from the donor nuclei, by injection or fusion, to produce an NT unit, containing cells which may be used to

obtain embryonic or stem-like cells and/or cell cultures. For example, the invention may involve the transplantation of an ungulate cell nucleus or ungulate cell into an enucleated oocyte of another species, e.g., another ungulate or non-ungulate, by injection or fusion, which cells and/or nuclei are combined to produce NT units and which are cultured under conditions suitable to obtain multicellular NT units, preferably comprising at least about 2 to 400 cells, more preferably 4 to 128 cells, and most preferably at least about 50 cells. The cells of such NT units may be used to produce embryonic or stem-like cells or cell colonies upon culturing.

However, the preferred embodiment of the invention comprises the production of human embryonic or stem-like cells by transplantation of the nucleus of a donor human cell or a human cell into an enucleated animal oocyte, preferably an ungulate oocyte, and most preferably a bovine enucleated oocyte.

In general, the embryonic or stem-like cells will be produced by a nuclear transfer process comprising the following steps:

- (i) obtaining desired human or animal cells to be used as a source of donor nuclei;
- (ii) obtaining oocytes from a suitable source, e.g. a mammal and most preferably an ungulate, e.g. bovine,
- (iii) enucleating said oocytes;
- (iv) transferring the human or animal cell or nucleus into the enucleated oocyte of an animal species different than the donor cell or nuclei, e.g., by fusion or injection;
- (v) culturing the resultant NT product or NT unit to produce multiple cell structures; and
- (vi) culturing cells obtained from said embryos to obtain embryonic or stem-like cells and stem-like cell colonies.

Nuclear transfer techniques or nuclear transplantation techniques are known in the literature and are described in many of the references cited in the Background of the Invention. See, in particular, Campbell et al, *Theriogenology*,

transfer and cloning, oocytes must generally be matured *in vitro* before these cells may be used as recipient cells for nuclear transfer, and before they can be fertilized by the sperm cell to develop into an embryo. This process generally requires collecting immature (prophase I) oocytes from animal ovaries, e.g.,
5 bovine ovaries obtained at a slaughterhouse and maturing the oocytes in a maturation medium prior to fertilization or enucleation until the oocyte attains the metaphase II stage, which in the case of bovine oocytes generally occurs about 18-24 hours post-aspiration. For purposes of the present invention, this period of time is known as the "maturation period." As used herein for calculation of time
10 periods, "aspiration" refers to aspiration of the immature oocyte from ovarian follicles.

Additionally, metaphase II stage oocytes, which have been matured *in vivo* have been successfully used in nuclear transfer techniques. Essentially, mature metaphase II oocytes are collected surgically from either non-superovulated or superovulated cows or heifers 35 to 48 hours past the onset of estrus or
15 past the injection of human chorionic gonadotropin (hCG) or similar hormone.

The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be significant to the success of NT methods. (See e.g., Prather et al., *Differentiation*, 48, 1-8, 1991). In general, successful mammalian
20 embryo cloning practices use the metaphase II stage oocyte as the recipient oocyte because at this stage it is believed that the oocyte can be or is sufficiently "activated" to treat the introduced nucleus as it does a fertilizing sperm. In domestic animals, and especially cattle, the oocyte activation period generally ranges from about 16-52 hours, preferably about 28-42 hours post-aspiration.

For example, immature oocytes may be washed in HEPES buffered hamster embryo culture medium (HECM) as described in Seshagine et al., *Biol. Reprod.*, 40, 544-606, 1989, and then placed into drops of maturation medium
25 consisting of 50 microliters of tissue culture medium (TCM) 199 containing 10% fetal calf serum which contains appropriate gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and estradiol under a
30

RESULTS

The cells grew to confluence, were implanted in the animals with the polymer scaffolds, and retrieved without complications. At retrieval, the implants maintained their initial size without any evidence of fibrosis.

5 Implants retrieved from the steer:

Histochemical and Immunocytochemical Analyses:

Histological examination demonstrated extensive vascularization throughout the implants and the presence of multinucleated giant cells were observed surrounding the polymer fibers. However, higher number of inflammatory cells were
10 present throughout the control allogeneic scaffolds. Histomorphomeric analysis of the explanted tissue (i.e., kidney, skeletal, heart, chondrocytes and keratinocytes) indicated that there was a statistically significant ($p < 0.05$; student's t-test) increase in lymphocytic infiltration of the control implants/constructs (non-cloned) versus the cloned tissue types (data not shown). This data suggests that the control grafts were
15 undergoing early graft rejection.

Engineered Kidney Tissue:

Histologically, glomeruli-like structures were observed in the retrieved scaffolds (data not shown). Histochemical analyses using periodic acid schiff identified renal tubular cells (data not shown). Immunocytochemical studies with
20 alkaline phosphatases antibodies confirmed the presence of proximal tubular cells. Studies using osteopontin antibodies were negative in the bovine tissue system.

Engineered Muscle Tissue:

Retrieved cardiac and skeletal muscle cell implants showed spatially oriented muscle fibers in each instance (data not shown). Immunocytochemical
25 analysis using tropomyosin antibodies identified skeletal muscle fibers within the construct (data not shown). Anti-troponin I stained cardiac muscle fibers positively (data not shown).

To prove that the mtDNA of the cloned tissues was from the recipient oocyte, the mtDNA of the nuclear donor and that of the cloned embryo were sequenced.
30 Sequence data confirmed that the mtDNAs were indeed different, particularly in the d-loop region where there were four different corresponding nucleotides in the cloned tissues in comparison with the nuclear donor.

oocyte will then be transferred into the perivitelline space of the enucleated oocyte used to produce the NT unit. The animal or human cell and the enucleated oocyte will be used to produce NT units according to methods known in the art. For example, the cells may be fused by electrofusion. Electrofusion is accomplished by providing a pulse of electricity that is sufficient to cause a transient breakdown of the plasma membrane. This breakdown of the plasma membrane is very short because the membrane reforms rapidly. Essentially, if two adjacent membranes are induced to breakdown and upon reformation the lipid bilayers intermingle, small channels will open between the two cells. Due to the thermodynamic instability of such a small opening, it enlarges until the two cells become one. Reference is made to U.S. Patent 4,997,384 by Prather et al., (incorporated by reference in its entirety herein) for a further discussion of this process. A variety of electrofusion media can be used including e.g., sucrose, mannitol, sorbitol and phosphate buffered solution. Fusion can also be accomplished using Sendai virus as a fusogenic agent (Graham, *Wister Inot. Symp. Monogr.*, 9, 19, 1969).

Also, in some cases (e.g. with small donor nuclei) it may be preferable to inject the nucleus directly into the oocyte rather than using electroporation fusion. Such techniques are disclosed in Collas and Barnes, *Mol. Reprod. Dev.*, 38:264-267 (1994), and incorporated by reference in its entirety herein.

Preferably, the human or animal cell and oocyte are electrofused in a 500 μm chamber by application of an electrical pulse of 90-120V for about 15 μsec , about 24 hours after initiation of oocyte maturation. After fusion, the resultant fused NT units are then placed in a suitable medium until activation, e.g., CR1aa medium. Typically activation will be effected shortly thereafter, typically less than 24 hours later, and preferably about 4-9 hours later.

The NT unit may be activated by known methods. Such methods include, e.g., culturing the NT unit at sub-physiological temperature, in essence by applying a cold, or actually cool temperature shock to the NT unit. This may be most conveniently done by culturing the NT unit at room temperature, which is

cold relative to the physiological temperature conditions to which embryos are normally exposed.

Alternatively, activation may be achieved by application of known activation agents. For example, penetration of oocytes by sperm during fertilization has been shown to activate prefusion oocytes to yield greater numbers of viable pregnancies and multiple genetically identical calves after nuclear transfer. Also, treatments such as electrical and chemical shock may be used to activate NT embryos after fusion. Suitable oocyte activation methods are the subject of U.S. Patent No. 5,496,720, to Susko-Parrish et al.

Additionally, activation may be effected by simultaneously or sequentially:

- (i) increasing levels of divalent cations in the oocyte, and
- (ii) reducing phosphorylation of cellular proteins in the oocyte.

This will generally be effected by introducing divalent cations into the oocyte cytoplasm, e.g., magnesium, strontium, barium or calcium, e.g., in the form of an ionophore. Other methods of increasing divalent cation levels include the use of electric shock, treatment with ethanol and treatment with caged chelators.

Phosphorylation may be reduced by known methods, e.g., by the addition of kinase inhibitors, e.g., serine-threonine kinase inhibitors, such as 6-dimethyl-amino-purine, staurosporine, 2-aminopurine, and sphingosine.

Alternatively, phosphorylation of cellular proteins may be inhibited by introduction of a phosphatase into the oocyte, e.g., phosphatase 2A and phosphatase 2B.

In the preferred embodiment, NT activation will be effected by briefly exposing the fused NT unit to a TL-HEPES medium containing $5\mu\text{M}$ ionomycin and 1 mg/ml BSA, followed by washing in TL-HEPES containing 30 mg/ml BSA within about 24 hours after fusion, and preferably about 4 to 9 hours after fusion.

The activated NT units may then be cultured in a suitable *in vitro* culture medium until the generation of embryonic or stem-like cells and cell colonies.

Culture media suitable for culturing and maturation of embryos are well known in the art. Examples of known media, which may be used for bovine embryo culture and maintenance, include Ham's F-10 + 10% fetal calf serum (FCS), Tissue Culture Medium-199 (TCM-199) + 10% fetal calf serum, Tyrodes-
5 Albumin-Lactate-Pyruvate (TALP), Dulbecco's Phosphate Buffered Saline (PBS), Eagle's and Whitten's media. One of the most common media used for the collection and maturation of oocytes is TCM-199, and 1 to 20% serum supplement including fetal calf serum, newborn serum, estrual cow serum, lamb serum or steer serum. A preferred maintenance medium includes TCM-199 with
10 Earl salts, 10% fetal calf serum, 0.2 MM Ma pyruvate and 50 µg/ml gentamicin sulphate. Any of the above may also involve co-culture with a variety of cell types such as granulosa cells, oviduct cells, BRL cells and uterine cells and STO cells.

Another maintenance medium is described in U.S. Patent 5,096,822 to
15 Rosenkrans, Jr. et al., which is incorporated herein by reference. This embryo medium, named CR1, contains the nutritional substances necessary to support an embryo.

CR1 contains hemicalcium L-lactate in amounts ranging from 1.0 mM to 10 mM, preferably 1.0 mM to 5.0 mM. Hemicalcium L-lactate is L-lactate with
20 a hemicalcium salt incorporated thereon. Hemicalcium L-lactate is significant in that a single component satisfies two major requirements in the culture medium: (i) the calcium requirement necessary for compaction and cytoskeleton arrangement; and (ii) the lactate requirement necessary for metabolism and electron transport. Hemicalcium L-lactate also serves as valuable mineral and energy
25 source for the medium necessary for viability of the embryos.

Advantageously, CR1 medium does not contain serum, such as fetal calf serum, and does not require the use of a co-culture of animal cells or other biological media, i.e., media comprising animal cells such as oviductal cells. Biological media can sometimes be disadvantageous in that they may contain
30 microorganisms or trace factors which may be harmful to the embryos and which

are difficult to detect, characterize and eliminate.

Examples of the main components in CR1 medium include hemicalcium L-lactate, sodium chloride, potassium chloride, sodium bicarbonate and a minor amount of fatty-acid free bovine serum albumin (Sigma A-6003). Additionally, a defined quantity of essential and non-essential amino acids may be added to the medium. CR1 with amino acids is known by the abbreviation "CR1aa."

CR1 medium preferably contains the following components in the following quantities:

| | |
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| sodium chloride | - 114.7 mM |
| potassium chloride | - 3.1 mM |
| sodium bicarbonate | - 26.2 mM |
| hemicalcium L-lactate | - 5 mM |
| fatty-acid free BSA | - 3 mg/ml |

In the preferred embodiment, the activated NT embryos unit will be placed in CR1aa medium containing 1.9 mM DMAP for about 4 hours followed by a wash in HECM and then cultured in CR1aa containing BSA.

For example, the activated NT units may be transferred to CR1aa culture medium containing 2.0 mM DMAP (Sigma) and cultured under ambient conditions, e.g., about 38.5°C, 5% CO₂ for a suitable time, e.g., about 4 to 5 hours.

Afterward, the cultured NT unit or units are preferably washed and then placed in a suitable media, e.g., CR1aa medium containing 10% FCS and 6 mg/ml contained in well plates which preferably contain a suitable confluent feeder layer. Suitable feeder layers include, by way of example, fibroblasts and epithelial cells, e.g., fibroblasts and uterine epithelial cells derived from ungulates, chicken fibroblasts, murine (e.g., mouse or rat) fibroblasts, STO and SI-m220 feeder cell lines, and BRL cells.

In the preferred embodiment, the feeder cells will comprise mouse embryonic fibroblasts. Means for preparation of a suitable fibroblast feeder layer is described in the example which follows and is well within the skill of the ordinary artisan.

The NT units are cultured on the feeder layer until the NT units reach a size suitable for obtaining cells which may be used to produce embryonic stem-like cells or cell colonies. Preferably, these NT units will be cultured until at least about 2 to 400 cells, more preferably about 4 to 128 cells, and most preferably at least about 50 cells. The culturing will be effected under suitable conditions, i.e., about 38.5°C and 5% CO₂, with the culture medium changed in order to optimize growth typically about every 2-5 days, preferably about every 3 days.

In the case of human cell/enucleated bovine oocyte derived NT units, sufficient cells to produce an ES cell colony, typically on the order of about 50 cells, will be obtained about 12 days after initiation of oocyte activation. However, this may vary dependent upon the particular cell used as the nuclear donor, the species of the particular oocyte, and culturing conditions. One skilled in the art can readily ascertain visually when a desired sufficient number of cells has been obtained based on the morphology of the cultured NT units.

After NT units of the desired size are obtained, the cells are mechanically removed from the zone and are then used to produce embryonic or stem-like cells and cell lines. This is preferably effected by taking the clump of cells which comprise the NT unit, which typically will contain at least about 50 cells, washing such cells, and plating the cells onto a feeder layer, e.g., irradiated fibroblast cells. Typically, the cells used to obtain the stem-like cells or cell colonies will be obtained from the inner most portion of the cultured NT unit which is preferably at least 50 cells in size. However, NT units of smaller or greater cell numbers as well as cells from other portions of the NT unit may also be used to obtain ES-like cells and cell colonies. The cells are maintained in the feeder layer in a suitable growth medium, e.g., alpha MEM supplemented with 10% FCS and 0.1 mM beta-mercaptoethanol (Sigma) and L-glutamine. The growth medium is changed as often as necessary to optimize growth, e.g., about every 2-3 days.

This culturing process results in the formation of embryonic or stem-like

hematopoietic stem cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of embryonic stem cells are known in the art as are suitable culturing conditions.

For example, Palacios et al, *Proc. Natl. Acad. Sci., USA*, 92:7530-7537 (1995) teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen, *J. Reprod. Fertil. Dev.*, 6:543-552 (1994) is a review article which references numerous articles disclosing methods for *in vitro* differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain et al, *Dev. Biol.*, 168:342-357 (1995) teaches *in vitro* differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem-like cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture medium, one skilled in the art may culture the subject embryonic or stem-like cells to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc.

The subject embryonic or stem-like cells may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated human cells are unparalleled. For example, human hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such

procedures are used to treat many diseases, e.g., late stage cancers such as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, e.g., bovine oocyte, obtaining embryonic or stem-like cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

Alternatively, adult somatic cells from a patient with a neurological disorder may be fused with an enucleated animal oocyte, e.g., a bovine oocyte, human embryonic or stem-like cells obtained therefrom, and such cells cultured under differentiation conditions to produce neural cell lines. Specific diseases treatable by transplantation of such human neural cells include, by way of example, Parkinson's disease, Alzheimer's disease, ALS and cerebral palsy, among others. In the specific case of Parkinson's disease, it has been demonstrated that transplanted fetal brain neural cells make the proper connections with surrounding cells and produce dopamine. This can result in long-term reversal of Parkinson's disease symptoms.

The great advantage of the subject invention is that it provides an essentially limitless supply of isogenic or syngenic human cells suitable for transplantation. Therefore, it will obviate the significant problem associated with current transplantation methods, i.e., rejection of the transplanted tissue which may occur because of host-vs-graft or graft-vs-host rejection. Conventionally, rejection is prevented or reduced by the administration of anti-rejection drugs such as cyclosporine. However, such drugs have significant adverse side-effects, e.g., immunosuppression, carcinogenic properties, as well as being very expensive. The present invention should eliminate, or at least greatly reduce, the need for anti-rejection drugs.

Other diseases and conditions treatable by isogenic cell therapy include,

by way of example, spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver diseases, i.e., hypercholesterolemia, heart diseases, cartilage replacement, burns, foot ulcers, gastrointestinal diseases, vascular diseases, kidney disease, urinary tract disease, and aging related diseases and conditions.

5 Also, human embryonic or stem-like cells produced according to the invention may be used to produce genetically engineered or transgenic human differentiated cells. Essentially, this will be effected by introducing a desired gene or genes, which may be heterologous, or removing all or part of an endogenous gene or genes of human embryonic or stem-like cells produced
10 according to the invention, and allowing such cells to differentiate into the desired cell type. A preferred method for achieving such modification is by homologous recombination because such technique can be used to insert, delete or modify a gene or genes at a specific cite or cites in the stem-like cell genome.

 This methodology can be used to replace defective genes, e.g., defective
15 immune system genes, cystic fibrosis genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc. For example, the gene encoding brain derived growth factor may be introduced into human embryonic or stem-like cells, the cells differentiated into neural cells and the cells transplanted into a
20 Parkinson's patient to retard the loss of neural cells during such disease.

 Previously, cell types transfected with BDNF varied from primary cells to immortalized cell lines, either neural or non-neural (myoblast and fibroblast) derived cells. For example, astrocytes have been transfected with BDNF gene using retroviral vectors, and the cells grafted into a rat model of Parkinson's
25 disease (Yoshimoto et al., *Brain Research*, 691:25-36, (1995)).

 This *ex vivo* therapy reduced Parkinson's-like symptoms in the rats up to 45% 32 days after transfer. Also, the tyrosine hydroxylase gene has been placed into astrocytes with similar results (Lundberg et al., *Develop. Neurol.*, 139:39-53 (1996) and references cited therein).

30 However, such *ex vivo* systems have problems. In particular, retroviral

vectors currently used are down-regulated *in vivo* and the transgene is only transiently expressed (review by Mulligan, *Science*, 260:926-932 (1993)). Also, such studies used primary cells, astrocytes, which have finite life span and replicate slowly. Such properties adversely affect the rate of transfection and
5 impede selection of stably transfected cells. Moreover, it is almost impossible to propagate a large population of gene targeted primary cells to be used in homologous recombination techniques.

By contrast, the difficulties associated with retroviral systems should be eliminated by the use of human embryonic or stem-like cells. It has been
10 demonstrated previously by the subject assignee that cattle and pig embryonic cell lines can be transfected and selected for stable integration of heterologous DNA. Such methods are described in commonly assigned U.S. Serial No. 08/626,054, filed April 1, 1996, incorporated by reference in its entirety. Therefore, using such methods or other known methods, desired genes may be
15 introduced into the subject human embryonic or stem-like cells, and the cells differentiated into desired cell types, e.g., hematopoietic cells, neural cells, pancreatic cells, cartilage cells, etc.

Genes which may be introduced into the subject embryonic or stem-like cells include, by way of example, epidermal growth factor, basic fibroblast
20 growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin-3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokine genes (interleukins, interferons, colony stimulating factors, tumor necrosis factors (alpha and beta), etc.), genes encoding therapeutic enzymes, etc.

Also, the subject embryonic or stem-like cells, preferably human cells,
25 may be used as an *in vitro* model of differentiation, in particular for the study of genes which are involved in the regulation of early development.

Also, differentiated cell tissues and organs using the subject embryonic or stem-like cells may be used in drug studies.

Further, the subject embryonic or stem-like cells may be used as nuclear
30 donors for the production of other embryonic or stem-like cells and cell colonies.

ionomycin (5 μ M; CalBiochem, La Jolla, CA) in TL-HEPES supplemented with 1 mg/ml BSA and then washed for five min in TL-HEPES supplemented with 30 mg/ml BSA. The NT units were then transferred into a microdrop of CR1aa culture medium containing 0.2 mM DMAP (Sigma) and cultured at 38.5°C 5% CO₂ for four to five hours. The NT units were washed and then placed in a CR1aa medium plus 10% FCS and 6 mg/ml BSA in four well plates containing a confluent feeder layer of mouse embryonic fibroblasts (described below). The NT units were cultured for three more days at 38.5°C and 5% CO₂. The culture medium was changed every three days until day 12 after the time of activation. At this time NT units reaching the desired cell number, i.e., about 50 cell number, were mechanically removed from the zona and used to produce embryonic cell lines. A photograph of an NT unit obtained as described above is contained in Figure 1.

Fibroblast feeder layer

Primary cultures of embryonic fibroblasts were obtained from 14-16 day old murine fetuses. After the head, liver, heart and alimentary tract were aseptically removed, the embryos were minced and incubated for 30 minutes at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells were plated in tissue culture flasks and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 μ l/ml). Three to four days after passage, embryonic fibroblasts, in 35 x 10 Nunc culture dishes (Baxter Scientific, McGaw Park, IL), were irradiated. The irradiated fibroblasts were grown and maintained in a humidified atmosphere with 5% CO₂ in air at 37°C. The culture plates which had a uniform monolayer of cells were then used to culture embryonic cell lines.

Production of embryonic cell line.

NT unit cells obtained as described above were washed and plated directly onto irradiated feeder fibroblast cells. These cells included those of the inner portion of the NT unit. The cells were maintained in a growth medium consisting of alpha MEM supplemented with 10% FCS and 0.1 mM beta-mercaptoethanol (Sigma). Growth medium was exchanged every two to three days. The initial colony was observed by the second day of culture. The colony was propagated and exhibits a similar morphology to previously disclosed mouse embryonic stem (ES) cells. Individual cells within the colony are not well defined and the perimeter of the colony is refractile and smooth in appearance. The cell colony appears to have a slower cell doubling time than mouse ES cells. Also, unlike bovine and porcine derived ES cells, the colony does not have an epithelial appearance thus far. Figures 2 through 5 are photographs of ES-like cell colonies obtained as described, *supra*.

15 Production of Differentiated Human Cells

The human embryonic cells obtained are transferred to a differentiation medium and cultured until differentiated human cell types are obtained.

RESULTS

Table 1. Human cells as donor nuclei in NT unit production and development.

TABLE 1

| Cell type | No. NT units made | No. NT units 2 cell stage (%) | No. NT units to 4 - 16 cell stage (%) | No. NT units to 16 - 400 cell stage (%) |
|------------------------|-------------------|-------------------------------|---------------------------------------|---|
| lymphocytes | 18 | 12 (67%) | 3 (17%) | 0 |
| oral cavity epithelium | 34 | 18 (53%) | 3 (9%) | 1 (3%) |

The one NT unit that developed a structure having greater than 16 cells was plated down onto a fibroblast feeder layer. This structure was attached to the feeder layer and started to propagate forming a colony with a ES cell-like morphology (*See, e.g., Figure 2*). Moreover, although the 4 to 16 cell stage structures were not used to try and produce an ES cell colony, it has been previously shown that this stage is capable of producing ES or ES-like cell lines (mouse, Eistetter et al., *Devel. Growth and Differ*, 31:275-282 (1989); *Bovine*, Stice et al., 1996)). Therefore, it is expected that 4 - 16 cell stage NT units should also give rise to embryonic or stem-like cells and cell colonies.

While the present invention has been described and illustrated herein by reference to various specific materials, procedures, and examples, it is understood that the invention is not restricted to the particular material, combinations of materials, and procedures selected for that purpose. Numerous variations of such details can be implied and will be appreciated by those skilled in the art.

CLAIMS:

1. A method of producing embryonic or stem-like cells comprising the following steps:
 - (i) inserting a desired human or mammalian cell or cell
5 nucleus into an enucleated animal oocyte, wherein such oocyte is derived from a different animal species than the human or mammalian cell under conditions suitable for the formation of a nuclear transfer (NT) unit;
 - (ii) activating the resultant nuclear transfer units;
 - (iii) culturing said activated nuclear transfer units until greater
10 than the 2-cell developmental stage; and
 - (iv) culturing cells obtained from said cultured NT units to obtain embryonic or stem-like cells.
2. The method of Claim 1, wherein the cell inserted into the enucleated animal oocyte is a human cell.
- 15 3. The method of Claim 2, wherein said human cell is an adult cell.
4. The method of Claim 2, wherein said human cell is an epithelial cell or lymphocyte.
5. The method of Claim 2, wherein the oocytes are obtained from a mammal.
- 20 6. The method of Claim 5, wherein the animal oocyte is obtained from an ungulate.
7. The method of Claim 6, wherein said ungulate is selected from the group consisting of bovine, ovine, porcine, equine, caprine, and buffalo.

8. The method of Claim 1, wherein the enucleated oocyte is matured prior to enucleation.

9. The method of Claim 1, wherein the fused nuclear transfer units are activated by exposure to ionomycin and DMAP.

5 10. The method of Claim 1, wherein the activated nuclear transfer units are cultured on a feeder layer culture.

11. The method of Claim 10, wherein the feeder layer comprises fibroblasts.

10 12. The method of Claim 1, wherein in step (iv) cells from a NT unit having 16 cells or more are cultured on a feeder cell layer.

13. The method of Claim 12, wherein said feeder cell layer comprises fibroblasts.

14. The method of Claim 13, wherein said fibroblasts comprise mouse embryonic fibroblasts.

15 15. The method of Claim 1, wherein the resultant embryonic or stem-like cells are induced to differentiate.

16. The method of Claim 2, wherein the resultant embryonic or stem-like cells are induced to differentiate.

20 17. The method of Claim 1, wherein fusion is effected by electrofusion.

18. Embryonic or stem-like cells obtained according to the method of Claim 1.

19. Human embryonic or stem-like cells obtained according to the method of Claim 2.

5 20. Human embryonic or stem-like cells obtained according to the method of Claim 3.

21. Human embryonic or stem-like cells obtained according to the method of Claim 4.

10 22. Human embryonic or stem-like cells obtained according to the method of Claim 6.

23. Human embryonic or stem-like cells obtained according to the method of Claim 7.

24. Differentiated human cells obtained by the method of Claim 16.

15 25. The differentiated human cells of Claim 24, which are selected from the group consisting of neural cells, hematopoietic cells, pancreatic cells, muscle cells, cartilage cells, urinary cells, liver cells, spleen cells, reproductive cells, skin cells, intestinal cells, and stomach cells.

20 26. A method of therapy which comprises administering to a patient in need of cell transplantation therapy isogenic differentiated human cells according to Claim 24.

27. The method of Claim 26, wherein said cell transplantation therapy

is effected to treat a disease or condition selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, ALS, spinal cord defects or injuries, multiple sclerosis, muscular dystrophy, cystic fibrosis, liver disease, diabetes, heart disease, cartilage defects or injuries, burns, foot ulcers,
5 vascular disease, urinary tract disease, AIDS and cancer.

28. The method of Claim 26, wherein the differentiated human cells are hematopoietic cells or neural cells.

29. The method of Claim 26, wherein the therapy is for treatment of Parkinson's disease and the differentiated cells are neural cells.

10 30. The method of Claim 26, wherein the therapy is for the treatment of cancer and the differentiated cells are hematopoietic cells.

31. The differentiated human cells of Claim 24, which contain and express an inserted gene.

15 32. The method of Claim 1, wherein a desired gene is inserted, removed or modified in said embryonic or stem-like cells.

33. The method of Claim 32, wherein the desired gene encodes a therapeutic enzyme, a growth factor or a cytokine.

34. The method of Claim 32, wherein said embryonic or stem-like cells are human embryonic or stem-like cells.

20 35. The method of Claim 32, wherein the desired gene is removed, modified or deleted by homologous recombination.

1 / 3

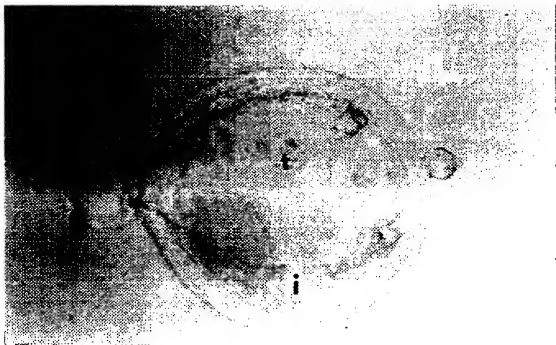


Fig. 1

2/3

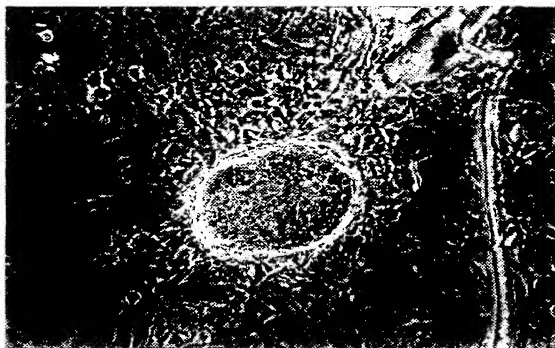


Fig. 2

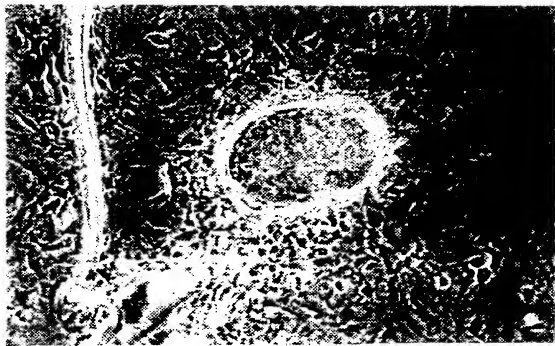


Fig. 3

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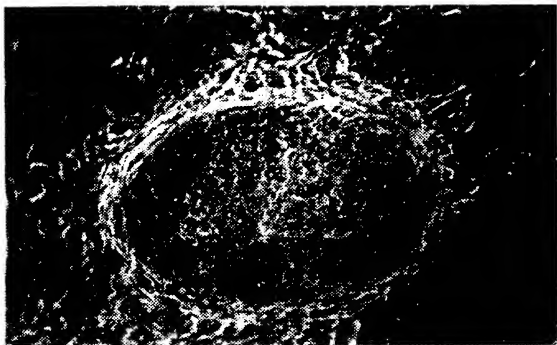


Fig. 4

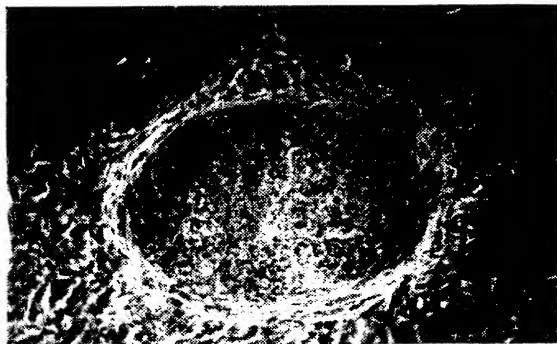


Fig. 5

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/12919

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00, 5/00; A01N 43/04; A61K 31/70
 US CL : 435/ 325, 366, 172.3, 69.1, 320.1; 424/92.31; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 325, 366, 172.3, 69.1, 320.1; 424/92.31; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| A | WOLFE ET AL. PREIMPLANTATION DEVELOPMENT OF EMBRYOS PRODUCED BY INTERGENERIC NUCLEAR TRANSPLANTATION. THERIOGENOLOGY. JANUARY 1990, VOL. 33, NO. 1, PAGE 350. SEE ENTIRE DOCUMENT. | 1-35 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | * later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
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| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

30 SEPTEMBER 1997

Date of mailing of the international search report

03 DEC 1997

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/12919

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, APS, BIOSIS, EMBASE, CAPLUS, WPIDS

search terms: nuclear transfer, embryonic stem-like cells, nuclear transplantation, xenotransplantation, cell transplantation therapy, donor cell nuclei, enucleated oocytes